



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 31/00	A2	(11) International Publication Number: WO 00/53169 (43) International Publication Date: 14 September 2000 (14.09.00)
<p>(21) International Application Number: PCT/US00/06482</p> <p>(22) International Filing Date: 10 March 2000 (10.03.00)</p> <p>(30) Priority Data: 60/124,135 12 March 1999 (12.03.99) US</p> <p>(71) Applicant (for all designated States except US): THE UNITED STATES OF AMERICA, represented by THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): MARCU, Monica, G. [CA/US]; 15600 Marathon Circle, Apt. 202, North Potomac, MD 20878 (US). NECKERS, Leonard, M. [US/US]; 5121 Wissioming Road, Bethesda, MD 20850 (US). SCHULTE, Theodor, W. [DE/US]; 4867 Battery Lane, Apt. 21, Bethesda, MD 20814 (US).</p> <p>(74) Agents: LARCHER, Carol et al.; Leydig, Voit & Mayer, Ltd., Two Prudential Plaza, Suite 4900, 180 North Stetson, Chicago, IL 60601-6780 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
(54) Title: METHOD OF INHIBITING A CHAPERONE PROTEIN		
<p>(57) Abstract</p> <p>The present invention provides a method of inhibiting binding of a chaperone protein with its client protein or client polypeptide. This method comprises contacting coumarin or a coumarin derivative with a chaperone protein, such that the coumarin or the coumarin derivative binds the chaperone protein, which inhibits the chaperone protein from binding its client protein or client polypeptide.</p>		

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METHOD OF INHIBITING A CHAPERONE PROTEIN

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method of inhibiting binding of a chaperone
5 protein with its client protein or client polypeptide.

BACKGROUND OF THE INVENTION

Chaperone proteins are proteins that mediate the correct assembly of other
proteins or polypeptides, called client proteins or client polypeptides, but are not
10 themselves part of the assembled structure. These proteins are involved in many
macromolecular assembly processes, such as the folding and refolding of proteins
during their synthesis and transport, as well as the association of polypeptides with
each other and other macromolecules to form oligomeric complexes. They have been
implicated in the folding of newly synthesized polypeptide chains, in the partial
15 unfolding or disassociation that may occur when a protein carries out its function, in
the transport of proteins across membranes, and in the repair of proteins partially
denatured by exposure to environmental stresses, such as high temperature.

Chaperone proteins act by binding noncovalently to specific structural features
in their target that are accessible only during assembly, and so inhibit unproductive
20 assembly pathways that would otherwise act as kinetic dead ends, producing an
incorrect, non-functional structure. Their function is required because many cellular
processes involving protein assembly carry an inherent risk of malfunction as a result
of the large number, variety, and flexibility of the noncovalent interactions that hold
proteins in their functional conformations. A number of essential cellular processes
25 involve the transient exposure of interactive protein surfaces to the intracellular
environment with the consequent risk of errors due to incorrect interactions. Such
processes include: protein synthesis, protein transport or translocation, protein
function, organelle biogenesis, and stress response. Chaperone proteins are located in
all parts of a cell where protein assembly occurs. Members of different classes of
30 chaperone proteins cooperate in mediating such processes as the transport of
polypeptides into mitochondria and their assembly into their final functional
conformations.

Recently, it has become clear that chaperone proteins interact with a variety of
proteins involved in cell proliferation. One such chaperone protein, heat shock
35 protein (Hsp) 90 is constitutively expressed at 2-10 fold higher levels in tumor cells
compared to their normal counterparts (see Ferrarini et al., *Int. J. Cancer* 51: 613-619
(1992)). Various compounds have been known to interfere with the chaperone protein

function of Hsp90. For example, the benzoquinone ansamycins, geldanamycin and herbimycin A, together with a non-related macrocyclic antibiotic, radicicol, have been shown to bind Hsp90 and to interfere with its function, including its function in tumor cell proliferation (see Whitesell et al., *Proc. Nat'l Acad. Sci. USA* 91: 8324-8328 (1994); Schulte et al., *Cell Stress and Chaperone Proteins* 3: 100-108 (1998); Johnson et al., *Mol. Endocrinol.* 9: 670-678 (1995); Sullivan et al., *J. Biol. Chem.* 272: 8007-8012 (1997)). Use of these compounds is not necessarily well-suited in clinical applications, as they display *in vivo* toxicity unrelated to their Hsp90 antagonism.

In view of the above, there exists a need for further methods utilizing chaperone protein antagonists. In particular, there is a need for methods utilizing clinically appropriate compounds that interfere with the function of chaperone proteins. The present invention provides such methods. These and other advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting binding of a chaperone protein with its client protein or client polypeptide. The method comprises contacting a chaperone protein with a coumarin or a coumarin derivative, such that the coumarin or the coumarin derivative binds the chaperone protein, which inhibits the chaperone protein from binding its client protein or client polypeptide. The client protein or the client polypeptide is inactive or less active subsequent to binding of the chaperone protein to coumarin or the coumarin derivative.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of inhibiting binding of a chaperone protein with its client protein or client polypeptide. The method comprises contacting a chaperone protein with a coumarin or a coumarin derivative, such that the coumarin or the coumarin derivative binds the chaperone protein, which inhibits the chaperone protein from binding its client protein or client polypeptide.

A chaperone protein in the context of the present invention can be any suitable chaperone protein or chaperone protein complex. Examples of chaperone and chaperone complex protein families include, but are not limited to, nucleoplasmins, chaperonins, heat shock proteins (Hsps), DnaJ protein, GrpE protein, SecB protein, signal recognition particle, prosequences, ubiquitinated proteins, PapD proteins, PrtM and PrsA, Lim protein, Rb protein, small heat shock proteins, ExbB protein, and prions.

Examples of heat shock protein complexes include, but are not limited to:

- 5 (1) the steroid receptor-associated Hsp90-containing intermediate folding complex, which functions in maturation of the progesterone receptor (PR) via an intermediate complex, Hsp70/Hsp90/p60^{Hop}-PR, such that, although the receptor is not yet competent to bind hormone, the intermediate complex is required for eventual maturation;
- (2) the Hsp70 family of chaperone proteins, which includes the cytosolic chaperone protein Hsc70, the heat stress-induced Hsp70, and the endoplasmic reticulum chaperone protein Grp78;
- 10 (3) co-chaperone protein p48^{Hsp}, which binds to Hsp70's adenosine triphosphatase (ATPase) domain and stabilizes its adenosine diphosphate (ADP)-bound state;
- (4) p60^{Hop}, which binds Hsp70 via an N-terminal tetratricopeptide repeat (TPR) motif, which is a degenerate sequence of 34 amino acids that often
15 occurs in tandem and appears to mediate protein-protein interaction, and to Hsp90 via a second TPR motif which is closer to the C-terminus and, when incubated with purified Hsp70 and Hsp90, forms a tripartite complex with the other two proteins;
- (5) p23, which is a highly acidic phosphoprotein that is a component of the
20 mature PR complex, is often found associated with Hsp90 and an immunophilin, even in the absence of PR, and, in addition to participating in steroid receptor-associated Hsp90 complexes, also can be found in Hsp90 complexes with hepatitis B virus reverse transcriptase, mutated p53, aryl hydrocarbon receptor, and heat shock transcription factor Hsf-1;
- 25 (6) immunophilins, which bind to Hsp90 via their TPR motifs and replace p60^{Hop} in the multichaperone protein complex, and also may have a transport function, since they have been found associated with both microtubules and nuclear structures;
- (7) two other proteins containing TPR motifs that have been shown to bind to
30 Hsp90, namely PP5, which is a serine/threonine phosphatase that contains 4 TPR motifs in its N-terminal domain and Mas70p, which is a component of the protein import machinery in the outer mitochondrial membrane that contains 7 TPR motifs (the existence of a Mas70p-Hsp90 complex suggests that Hsp90 may participate in protein movement into
35 mitochondria); and

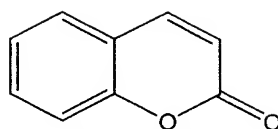
(8) p50^{Cdc37}, which replaces immunophilin in Hsp90 complexes with kinases such as Cdk4, v-Src and Raf-1 and does not possess TPR motifs, but binds to a site close to Hsp90's TPR binding domain.

Preferably, the chaperone protein is Hsp90, which is one of the most abundant proteins in eukaryotic cells, comprising 1-2% of total cellular protein, even under non-stress conditions. In mammalian cells, there are two Hsp90 isomers in the cytosol, Hsp90 α and Hsp90 β in humans and Hsp86 and Hsp84 in mice, with a third homologue, glucose regulated protein 94 (Grp94), localized primarily in the endoplasmic reticulum. An additional truncated, cytosolic member of the family, designated Hsp75, has recently been identified. Functional analysis has revealed that Hsp90 is composed of three primary domains: well-conserved amino and carboxyl terminal regions separated by a charged domain (see Scheibel et al., *J. Biol. Chem.*, 272:18608-18613 (1997); Scheibel et al., *Proc Natl Acad Sci U S A* 95:1495-1499 (1998)).

Hsp90 is required for full activity of several "ligand-dependent" transcription factors, including members of the steroid receptor family, the aryl hydrocarbon receptor and the retinoid receptor. "Ligand-independent" transcription factors that bind Hsp90 include MyoD, Hsf-1, mutated p53 and hypoxia inducible factor 1- α . Hsp90 or Grp94 also has been demonstrated to be necessary for proper function and correct cellular localization of a wide variety of tyrosine and serine/threonine kinases, including members of the Src family, oncogenic epidermal growth factor (EGF) receptor-related tyrosine kinase p185^{erbB2}, cyclin-dependent kinase 4 (Cdk4), cell cycle-associated kinase Wee1, and serine-threonine kinase Raf-1, which is a member of the mitogen-activated protein (MAP) kinase pathway.

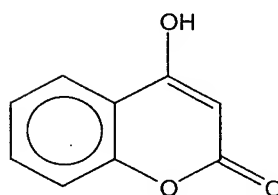
The "coumarin or a coumarin derivative" is any suitable member of the coumarin family. This family of compounds has a 2H-1-benzopyran-2-one core (molecular formula C₉H₆O₂), which is shown below (see DeGarmo, *Coumarin*, in ECT 1st ed., vol. 4, 588-593; 2nd ed., vol. 6, 425-433, (Monsanto Chemical Co.); Sethna et al., *Chem. Rev.* 36: 27 (1945)). Derivatives include, e.g., alkyl, hydroxy, and methoxy derivatives, as well as more complicated derivatives, such as, e.g., 3,4-dihydrocoumarin, 6-methylcoumarin, umbelliferone (7-hydroxycoumarin), 4-hydroxycoumarin (shown below), dicumarol (shown below), warfarin (3-substituted 4-hydroxycoumarins, an example of which is shown below), phenprocoumon (shown below), coumarone (benzofuran), and coumarone-inden \bar{e} resins.

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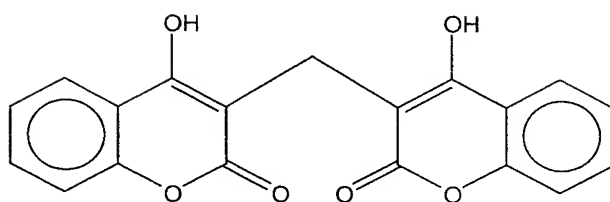
Coumarin

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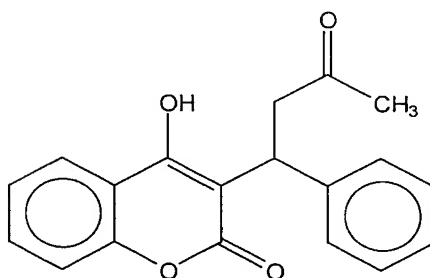
4-Hydroxycoumarin

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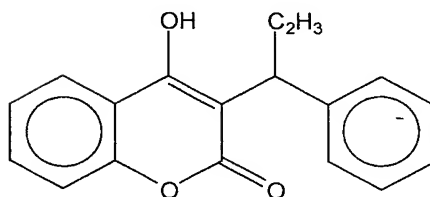


Dicumarol

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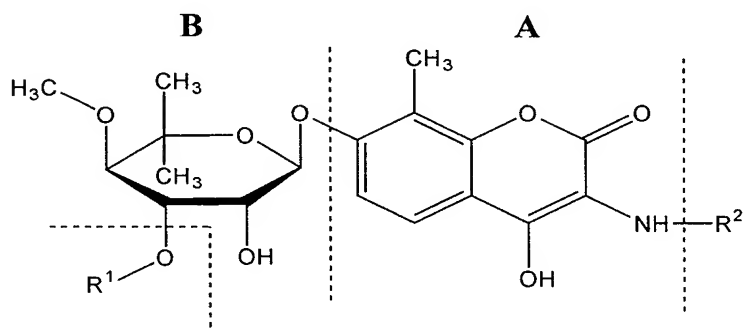


Warfarin



Phenprocoumon

Preferably, coumarin or a coumarin derivative is a coumarin antibiotic. Coumarin antibiotics have a 4-hydroxy-8-methylcoumarin core (A) and a noviose sugar moiety (B), shown below (see Finland & Nichols, *Anti-Biot. Chemother.* 4: 1954-68 (1957); Godfrey & Price, *Structure-Activity Relationships in Coumermycins*, in *Structure-Activity Among the Semisynthetic Antibiotics* 653-718 (D. Perlman, ed. 1977)).



R^1 and R^2 can be any suitable substituent. Examples of suitable substituents for R^1 include H, alkyl, haloalkyl, alkylthio, alkenyl, alkynyl, alkoxy, haloalkoxy, alkylamino, dialkylamino, cycloalkyl, aryl, aralkyl, heterocycloalkyl, heteroaryl, or $O=C-R^3$. R^3 can be any suitable substituent, e.g., H, alkyl, haloalkyl, alkylthio, alkenyl, alkynyl, alkoxy, haloalkoxy, alkylamino, dialkylamino, cycloalkyl, aryl, aralkyl, heterocycloalkyl, heteroaryl, or NR^4 . R^4 can be any suitable substituent, e.g., H, alkyl, haloalkyl, alkylthio, alkenyl, alkynyl, alkoxy, haloalkoxy, alkylamino, dialkylamino, cycloalkyl, aryl, aralkyl, heterocycloalkyl, or heteroaryl. Preferably, R^3 is a substituted heteroaryl. Examples of suitable substituents for R^2 include H, alkyl, haloalkyl, alkylthio, alkenyl, alkynyl, alkoxy, haloalkoxy, alkylamino, dialkylamino, cycloalkyl, aryl, aralkyl, heterocycloalkyl, heteroaryl, or $O=C-R^5$. R^5 can be any suitable substituent, e.g., H, alkyl, haloalkyl, alkylthio, alkenyl, alkynyl, alkoxy, haloalkoxy, alkylamino, dialkylamino, cycloalkyl, aryl, aralkyl, heterocycloalkyl, or heteroaryl. Preferably, R^5 is an aryl, more preferably, monocyclic, and most preferably, 6-membered and substituted with, e.g., halogen (e.g., fluorine, chlorine, or bromine), alkyl, haloalkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, nitro, cyano, hydroxy, amino, thio, alkylthio, or acyl. These substituents may, themselves, be substituted or unsubstituted.

As utilized herein, the term "alkyl" means a straight-chain or branched-chain alkyl radical which, unless otherwise specified, contains from about 1 to about 20 carbon atoms chain, preferably from about 1 to about 10 carbon atoms, more preferably from about 1 to about 8 carbon atoms, and most preferably from about 1 to about 6

carbon atoms. Examples of such alkyl radicals include methyl, ethyl, propyl (i.e., *n*- or isopropyl), butyl (i.e., *n*-, *sec*-, iso, or *tert*-butyl), pentyl, isoamyl, hexyl, octyl, dodecanyl, and the like.

5 The term “haloalkyl” means alkyl, as defined herein, wherein a hydrogen atom is replaced by a halogen (e.g., chlorine, fluorine, or bromine).

The term “alkylthio” means alkyl, as defined herein, which has a sulfur substituent. Example of alkylthios include methanethiol, ethanethiol, and the like.

10 The term “alkenyl” means a straight-chain or branched-chain alkenyl radical, which has one or more double bonds and, unless otherwise specified, contains from about 2 to about 20 carbon atoms, preferably from about 2 to about 10 carbon atoms, more preferably from about 2 to about 8 carbon atoms, and most preferably from about 2 to about 6 carbon atoms. Examples of alkenyl radicals include vinyl, allyl, 1,4-butadienyl, isopropenyl, and the like.

15 The term “alkynyl” means a straight-chain or branched-chain alkynyl radical, which has one or more triple bonds and contains from about 2 to about 20 carbon atoms, preferably from about 2 to about 10 carbon atoms, more preferably from about 2 to about 8 carbon atoms, and most preferably from about 2 to about 6 carbon atoms. Examples of alkynyl radicals include ethynyl, propynyl (propargyl), butynyl, and the like.

20 The term “alkoxy” means a straight-chain or branched-chain alkoxy radical, which has one or more ether groups of the general formula O–R and contains from about 2 to about 20 carbon atoms, preferably from about 2 to about 10 carbon atoms, more preferably from about 2 to about 8 carbon atoms, and most preferably from about 2 to about 6 carbon atoms. Examples of alkoxy radicals include methoxy, ethoxy, and
25 the like.

The term “haloalkoxy” means alkoxy, as defined herein, wherein a hydrogen atom is replaced by a halogen (e.g., chlorine, fluorine, or bromine).

30 The terms “alkylamino” and “dialkylamino” mean an alkyl amine or a dialkyl amine radical, wherein the term “alkyl” is defined as above. Examples of alkylamino radicals include methylamino (NHCH₃), ethylamino (NHCH₂CH₃), propylamino (i.e., *n*- or isopropylamino), butylamino (i.e., *n*-, iso, *sec*-, or *tert*-butylamino), *n*-hexylamino, and the like. Examples of dialkylamino radicals include dimethylamino (N(CH₃)₂), diethylamino (N(CH₂CH₃)₂), dipropylamino (i.e., di-*n*- or di-isopropylamino), dibutylamino (i.e., di-*n*-, di-iso, di-*sec*-, or di-*tert*-butylamino), di-*n*-hexylamino, and
35 the like.

The term “cycloalkyl” means a monocyclic alkyl radical, or a polycyclic alkyl which comprises one or more alkyl carbocyclic rings, which can be the same or

different when the polycyclic radical has 3 to about 10 carbon atoms in the carbocyclic skeleton of each ring. Preferably, the cycloalkyl has from about 4 to about 7 carbon atoms, more preferably from about 5 to about 6 carbons atoms. Examples of monocyclic cycloalkyl radicals include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclodecyl, and the like. Examples of polycyclic cycloalkyl radicals include decahydronaphthyl, bicyclo[5.4.0]undecyl, adamantyl, and the like.

The term "aryl" refers to an aromatic carbocyclic radical, as commonly understood in the art, and includes monocyclic and polycyclic aromatics such as, e.g., phenyl and naphthyl radicals, which radicals are, unless indicated otherwise, optionally substituted with one or more substituents selected from the group consisting of a halogen, an alkyl, alkoxy, amino, cyano, nitro, and the like. Preferably, the aryl has one or more six-membered carbocyclic rings including, e.g., phenyl, naphthyl, and biphenyl, and are optionally substituted as set forth herein.

The term "aralkyl" means alkyl as defined herein, wherein an alkyl hydrogen atom is replaced by an aryl as defined herein. Examples of aralkyl radicals include benzyl, phenethyl, 1-phenylpropyl, 2-phenylpropyl, 3-phenylpropyl, 1-naphthylpropyl, 2-naphthylpropyl, 3-naphthylpropyl, 3-naphthylbutyl, and the like.

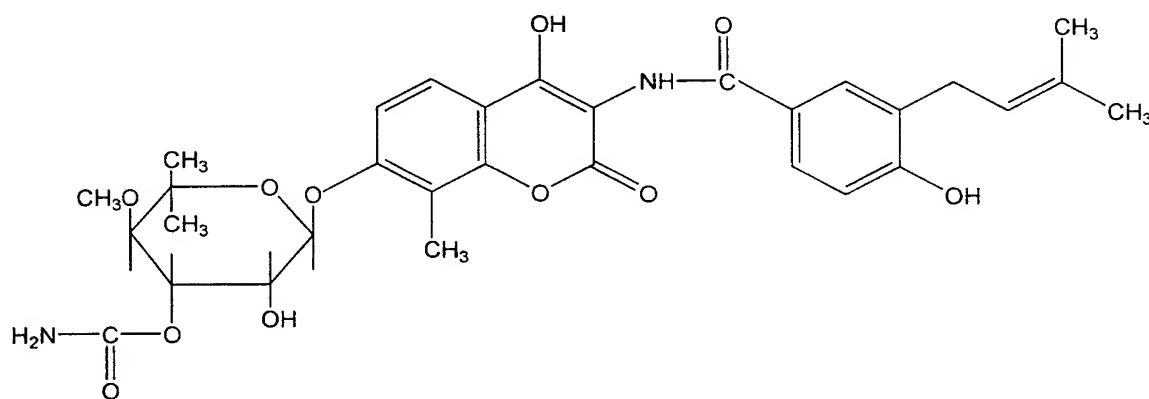
The terms heterocycle and heterocyclic refers to both heterocycloalkyls and heteroaryls. The term "heterocycloalkyl" means a cycloalkyl radical as defined herein (including polycyclics), wherein at least one carbon of a carbocyclic ring is substituted with a heteroatom such as, e.g., O, N, or S. The heterocycloalkyl optionally has one or more double bonds within a ring, but is not necessarily aromatic. The heterocycloalkyl preferably has 3 to about 10 atoms (members) in the carbocyclic skeleton of each ring, preferably from about 4 to about 7 atoms, more preferably from about 5 to about 6 atoms. Examples of heterocycloalkyl radicals include epoxy, aziridyl, oxetanyl, tetrahydrofuranyl, ribose, dihydrofuranyl, piperidinyl, piperazinyl, pyranyl, morpholinyl, and the like.

The term "heteroaryl" means a radical defined by an aromatic heterocyclic ring as commonly understood in the art, including monocyclic radicals such as, e.g., imidazole, thiazole, pyrazole, pyrrole, furane, pyrazoline, thiophene, oxazole, isoxazole, pyridine, pyridone, pyrimidine, cytosine, 5-methylcytosine, thymine, pyrazine, and triazine radicals, and polycyclics such as, e.g., quinoline, isoquinoline, indole, purine, adenine, guanine, N⁶-methyladenine, and benzothiazole radicals, which heteroaryl radicals are optionally substituted with one or more substituents selected from the group consisting of a halogen, an alkyl, alkoxy, an amino, a cyano, a nitro, and the like. It will also be appreciated that heteroaryls, as defined herein, are not necessarily "aromatic" in the same context as phenyl is aromatic, although heteroaryls

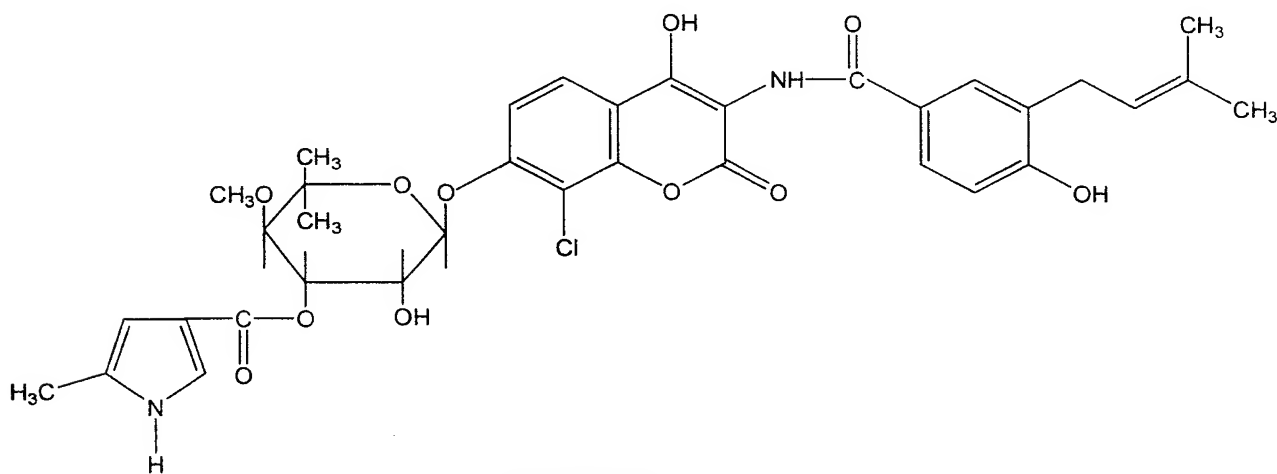
nonetheless demonstrate physical and chemical properties associated with aromaticity, as the term is understood in the art.

Any of the above may be substituted or unsubstituted with any suitable substituent. Examples of suitable substituents include halogen (e.g., fluorine, chlorine, or bromine), alkyl, haloalkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, nitro, cyano, hydroxy, amino, thio, alkylthio, or acyl. These substituents may, themselves, be substituted or unsubstituted.

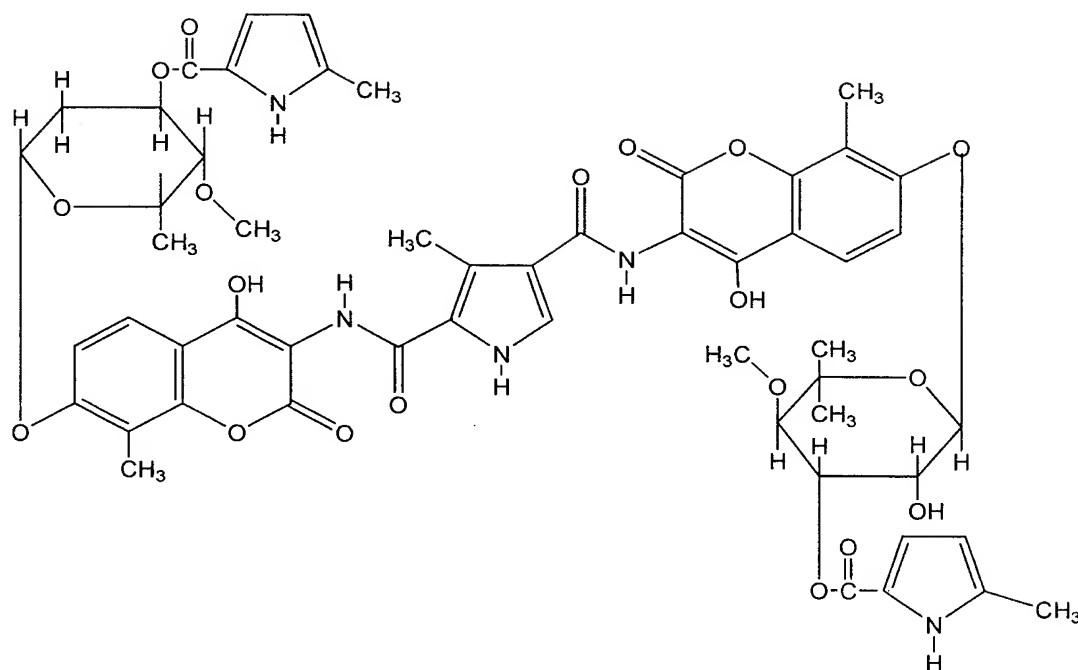
More preferably, the coumarin antibiotic is novobiocin, chlorobiocin, or coumermycin A1, which are shown below.



Novobiocin



Chlorobiocin



Coumermycin A1

Most preferably, the coumarin antibiotic is novobiocin, a well-studied
 5 antibiotic whose pharmacokinetics and toxicity profile are clearly understood. Doses of
 4 g/day (well below the maximum tolerated dose) yield a plasma level ≥ 200 -
 300 $\mu\text{g/ml}$, 2 hrs after post-administration, corresponding to a 300-500 μM drug
 concentration (see Drusano et al., *Antimicrob. Agents Chemother.* 30: 42-45 (1986);
 Eder et al., *J. Clin. Invest.* 79: 1524-1528 (1987)).

10 The contacting of coumarin or a coumarin derivative with the chaperone
 protein can be carried out in any suitable manner. For example, coumarin or a
 coumarin derivative can be contacted with the chaperone protein by *in vivo* or *ex vivo*
 administration. Preferably, coumarin or a coumarin derivative is administered *in vivo*
 to a mammal, more preferably, coumarin or a coumarin derivative is administered *in*
 15 *vivo* to a human.

To facilitate administration of coumarin or a coumarin derivative, both *in vivo*
 and *ex vivo*, it can be formulated into a suitable composition. Generally, such
 compositions (e.g., pharmaceutical compositions) include the active ingredient (i.e.,
 coumarin or a coumarin derivative) and an acceptable carrier (e.g. a
 20 pharmacologically acceptable carrier). Such compositions can be suitable for delivery
 of the active ingredient to a patient for medical application, and can be manufactured
 in a manner that is itself known, e.g., by means of conventional mixing, dissolving,

granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more pharmacologically
5 (e.g., physiologically) acceptable carriers comprising excipients, as well as optional auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Thus, for injection, the active ingredient can be formulated in aqueous solutions, preferably in physiologically compatible buffers. For
10 transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral administration, the active ingredient can be combined with carriers suitable for inclusion into tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like. For administration by inhalation, the active ingredient is
15 conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant. The active ingredient can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Such compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents
20 such as suspending, stabilizing and/or dispersing agents. Other pharmacological excipients are known in the art.

The coumarin or coumarin derivative can be administered in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects,
25 each unit containing a predetermined quantity, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle. The specifications for the unit dosage forms of the present invention depend on the particular coumarin or coumarin derivative employed and the effect to be achieved, as
30 well as the pharmacodynamics associated with each in the host. The dose administered should be an "effective amount" or an amount necessary to achieve an "effective level" in the individual patient. Since the effective level is used as the preferred endpoint for dosing, the actual dose and schedule can vary, depending on interindividual differences in pharmacokinetics, drug distribution, and metabolism.

35 One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired effective level in the individual patient. Many of the

coumarin or coumarin derivatives are well-known compounds with clearly established effective amounts required to achieve effective levels. However, one skilled in the art also can readily determine and use an appropriate indicator of the effective level of the compounds of the present invention by a direct (e.g., analytical chemical analysis) or indirect analysis of appropriate patient samples (e.g., blood and/or tissues) to determine the suitable dosage required for any given coumarin or coumarin derivative administered.

The interaction between the chaperone protein and coumarin or a coumarin derivative is such that the chaperone protein does not bind or binds with less affinity to its client protein or client polypeptide. Such interference with binding can be accomplished by any suitable method. For example, the chaperone protein and coumarin or a coumarin derivative may interact by binding covalently or non-covalently. If non-covalent, the binding can be through hydrogen bonding, ionic bonding, hydrophobic or van der Waals interactions, or any other appropriate type of binding. Preferably, the binding is through non-covalent binding, more preferably, hydrogen or hydrophobic binding. In the preferred embodiment wherein the chaperone protein is Hsp90 and the coumarin or coumarin derivative is novobiocin, interaction is such that novobiocin binds a carboxyl-terminal region of Hsp90, which contains an adenosine triphosphate (ATP)-binding domain.

A client protein or client polypeptide in the context of the present invention can be any suitable client protein or client polypeptide. Client proteins or client polypeptides include, but are not limited to, various transcription factors, including steroid hormone receptors, aryl hydrocarbon receptor, v-ErbA, retinoid receptor, Sim, Myo D1, Hsf-1, mutated p53, various protein kinases, and various other proteins, such as cytoskeletal proteins, calmodulin, G protein $\beta\gamma$ -subunits, Proteasome, Hepatitis B virus reverse transcriptase, tumor necrosis factor (TNF) receptors and retinoblastoma protein. *See generally* Thormeyer & Baniahmad, *Int. J. Mol. Med.* 4(4):351-58 (1999) (vErbA); Moffett & Pelletier, *FEBS Lett.* 466(1):80-6 (2000) (Sim); Jones et al., *B. Biol. Sci.* 326(1235):277-84 (1990) (Myo D1); Green et al., *Mol. Cell Biol.* 15:3354-62 (1995) (Hsf-1). Examples of steroid hormone receptors include the glucocorticoid receptor, the progesterone receptor, the estrogen receptor, the androgen receptor, and the mineralocorticoid receptor. Cytoskeletal proteins include actin, tubulin, and intermediate filaments.

Preferably, the client protein or the client polypeptide is a protein kinase. More preferably, the protein kinase is a tyrosine kinase or a serine/threonine kinase. Serine/Threonine kinases include the Raf family of kinases, MEK (MAP/ERK (extracellular signal-regulated kinase)), heme regulated E1F-2a kinase, calmodulin

dependent eukaryotic elongation factor (eEF)-2 kinase, and casein kinase. *See generally* Weber et al., *Oncogene* 19(2):169-76 (2000) (Raf family); Bommhardt et al., *J Immunol.* 164(5):2326-37 (2000) (MEK). Tyrosine kinases include HER-2/neu- (also known as c-erbB2) encoded p185 (p185^{erbB2}), the Src family kinases (p60^{v-src} or p60^{c-src}), Wee1, Sevenless, and Fps/Fes. *See generally* Hung & Lau, *Semin. Oncol.* 26 (4 Suppl 12):51-9 (1999) (HER-2/neu-encoded p185); Schwartzberg, *Oncogene* 17:1463-8 (1998) (Src family kinases); Pendergast, *Curr. Opin. Cell Biol.* 8(2):174-81 (1996) (Wee1); Smithgall et al., *Crit. Rev. Oncogene* 9(1):43-62 (1998) (Fps/Fes). Most preferably, the client protein or the client polypeptide is p185^{erbB2}, p60^{v-src} or Raf-1. Alternatively, the client protein or the client polypeptide is preferably a mutated p53 protein.

After binding of the chaperone protein to the coumarin or the coumarin derivative, the client protein or the client polypeptide is generally inactive or less active. The client protein or the client polypeptide can be inactive or less active through any suitable method. For example, the client protein or the client polypeptide can be degraded by cellular machinery because it is not coupled with its chaperone protein, or it can have an incorrect conformation that distorts its active or recognition site and interferes with the polypeptide performing its designated function. The client protein or the client polypeptide can also, e.g., be located in an inappropriate area of the cell, which prevents it from performing its designated function. Preferably, the client protein or client polypeptide is degradation.

The present invention is further described in the following examples. These examples serve only to illustrate the invention and are not intended to limit the scope of the invention in any way.

25

EXAMPLES

Example 1

This example demonstrates binding of a chaperone protein to coumarin or a coumarin derivative. In addition, this example illustrates that the incubation of the chaperone protein with various coumarin derivatives inhibits subsequent binding of the chaperone protein to its client protein or client polypeptide.

To demonstrate binding of a chaperone protein to novobiocin (a coumarin antibiotic), the novobiocin was first immobilized on sepharose (see Staudenbauer & Orr, *Nucleic Acids Res.* 9: 3589-3603 (1981)). A chaperone protein, either pure Hsp90, or a solution containing Hsp90 in a cell lysate, was subsequently incubated with the immobilized novobiocin. The cell lysate was preincubated with various members of the coumarin family of antibiotics, namely novobiocin, chlorobiocin, or coumermycin A1,

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and also ATP to determine their ability to inhibit binding of the Hsp90. The amount of Hsp90 bound to the immobilized novobiocin was analyzed by SDS-PAGE followed by silver staining using, e.g., a commercially available kit from BioRad or Western blotting with appropriate antibodies, e.g., SPA830 (StressGen Biotechnology, Vancouver,
5 Canada).

Immobilized novobiocin bound in a hydrophobic manner to both of the pure Hsp90 and the Hsp90 present in cell lysate. Pre-incubation of the cell lysate with excess soluble novobiocin, chlorobiocin, coumermycin A1, or ATP inhibited, in a dose-dependent manner, subsequent Hsp90 binding to immobilized novobiocin, as
10 determined by Western blotting. Soluble novobiocin inhibited Hsp90 binding to immobilized novobiocin at about 8 mM. Chlorobiocin and coumermycin A1 inhibited Hsp90 binding to immobilized novobiocin at about 0.5 mM, while ATP inhibited Hsp90 binding between about 10 and 15 mM, as demonstrated by silver staining.

These data demonstrate that novobiocin, a coumarin derivative, binds to
15 chaperone proteins such as Hsp90 and that subsequent Hsp90-binding can be inhibited by contact with the coumarin derivatives novobiocin, chlorobiocin, and coumermycin A1, as well as ATP.

Example 2

20 This example demonstrates *in vivo* depletion of client protein or client polypeptide through contact of a coumarin or a coumarin derivative with a chaperone protein.

To determine the depletion of Hsp90 client polypeptides, commercially available SKBR3 cells (American Type Culture Collection, Rockville, MD) and *v-src*-3T3
25 fibroblasts (National Cancer Institute, Rockville, MD) were treated with increasing concentrations of novobiocin, a coumarin antibiotic. The levels of the various Hsp90 client polypeptides were assayed by Western blotting: the membranes were first probed with an appropriate primary antibody (i.e., p185^{erbB2}, pp60^{v-src}, and p53 antibodies, Oncogene Research Products/Calbiochem, Cambridge, MA; gelsolin antibody, Sigma;
30 Grp78 and Raf-1 antibodies, Santa Cruz Biotechnology, Santa Cruz, CA; and scinderin antibody, a gift from Dr. J.M. Trifaró, University of Ottawa, Canada), followed by a secondary antibody conjugated to horseradish peroxidase, and the signal detected by chemiluminescent reagents.

As a control, the steady-state levels of scinderin, an actin-associated protein, and
35 BiP (Grp78), an Hsp70 family chaperone protein localized to the endoplasmic reticulum, were assayed using the same method described above. General interference

with protein synthesis was also determined after overnight cyclohexamide treatment of SKBR3 cells.

The level of p185^{erbB2} protein, an Hsp90 client polypeptide, was reduced by 80% relative to untreated controls after overnight treatment with 800 μ M novobiocin, and by 40% compared to controls after overnight treatment with 300 μ M novobiocin. Hsp90 client polypeptides Raf-1, p60^{v-src}, and mutant p53 protein levels were also significantly reduced after overnight treatment with novobiocin. In v-src-transformed 3T3 fibroblasts, the level of p60^{v-src} protein was reduced by 50% after exposure to 500 μ M novobiocin. Novobiocin also significantly depleted mutant p53 protein in SKBR3 cells, while depleting Raf-1 protein to an undetectable level in the same cells. As a control, the steady-state levels of scinderin, an actin-associated protein, and BiP (Grp78), an Hsp70 family chaperone protein localized to the ER, were not altered by the doses and exposure times of novobiocin used. General interference with protein synthesis is also not likely since overnight cyclohexamide treatment of SKBR3 cells did not significantly affect the steady state levels of the proteins affected by novobiocin.

Thus, these data demonstrate that contact of novobiocin, a coumarin derivative, with the chaperone protein Hsp90 inhibits Hsp90-binding with p185^{erbB2}, an Hsp90 client protein, thereby resulting in the subsequent inactivation, and specifically depletion, of the client protein p185^{erbB2}.

Example 3

This example demonstrates that contacting coumarin or a coumarin derivative with a chaperone protein decreases client protein or client polypeptide levels in normal human peripheral blood mononuclear cells (PBMC).

To determine the decrease in Raf-1 (an Hsp90 client protein) after treatment with novobiocin (a coumarin antibiotic), PBMC were isolated from human blood and cultured, according to standard protocol, with varying concentrations of novobiocin for 14 hrs. Levels of Raf-1 protein were determined using Western blotting, as described in Example 2. A control protein, gelsolin, was also assayed using the same method. Cell viability was assessed by the ability of cells to take up a vital stain (e.g., trypan blue), which is ordinarily excluded by viable cells.

The Hsp90 client Raf-1 protein was found to be depleted in a dose-dependent manner by incubation with novobiocin, a coumarin derivative, such that it was almost completely depleted at a concentration of about 0.8 mM novobiocin. Other protein levels remained unaltered, even at the highest drug concentration tested. PBMC remained viable, as assayed by trypan blue.

Example 4

This example demonstrates that *in vivo* administration of coumarin or a coumarin derivative affects client protein or client polypeptide levels in murine splenocytes.

5 To determine the level of Raf-1 protein (an Hsp-90 client polypeptide), normal C57Bl6 mice received intraperitoneal injections (100 mg/kg) of novobiocin (a coumarin antibiotic) at 12 hr intervals for 5 days. Animals were sacrificed 3 hrs after the last injection. The spleens were removed, cells were lysed and total protein was assayed by the Bradford method using, e.g., a commercially available kit from BioRad. At this
10 dose, the serum level of novobiocin has been reported to vary between 100 and 450 µg/ml during the first hr, with the plasma clearance half-life of the drug approximately 80 min. in mice (see Eder et al., *Cancer Res.* 51: 510-513 (1991)). Raf-1 levels were determined by Western blotting, as described in Example 2. A control protein, gelsolin, was also assayed using the same method. Optical density of the Raf-1
15 specific bands was determined using NIH Image software.

After 5 days on this regimen, mice receiving novobiocin had significantly lower levels of splenocyte Raf-1 protein than did controls. On average, Raf-1 protein in the novobiocin-treated group was reduced by 44% as compared to controls. In 7 of 10 treated mice, the mean splenocyte Raf-1 protein level was reduced even further, to 29%
20 of control. Splenocyte gelsolin remained unchanged in all treated mice.

These data demonstrate that *in vivo* treatment with novobiocin significantly reduces the levels of the client protein Raf-1 in the tissue targeted. Presumably, the reduced levels were due to contact between novobiocin, a coumarin derivative, and the chaperone protein Hsp90.

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All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred
30 embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of inhibiting binding of a chaperone protein with its client protein or client polypeptide, wherein the method comprises contacting a chaperone protein with coumarin or a coumarin derivative, such that the coumarin or the coumarin derivative binds the chaperone protein, which binding inhibits the chaperone protein from binding its client protein or client polypeptide.
2. The method of claim 1, wherein the chaperone protein is heat shock protein (Hsp) 90.
3. The method of claim 1, wherein the coumarin or coumarin derivative is a coumarin antibiotic.
4. The method of claim 3, wherein the coumarin antibiotic is chlorobiocin or coumermycin A1.
5. The method of claim 3, wherein the coumarin antibiotic is novobiocin.
6. The method of claim 2, wherein the coumarin or coumarin derivative is novobiocin.
7. The method of claim 6, wherein novobiocin binds a carboxyl-terminal region of Hsp90.
8. The method of claim 1, wherein the client protein or the client polypeptide is a tyrosine or serine/threonine kinase.
9. The method of claim 8, wherein the client protein or the client polypeptide is tyrosine kinase p185^{erbB2} or p60^{v-src}.
10. The method of claim 8, wherein the client protein or the client polypeptide is serine/threonine kinase Raf-1.
11. The method of claim 1, wherein the client protein or the client polypeptide is a mutated p53 protein.
12. The method of claim 1, wherein the client protein or the client polypeptide is inactive subsequent to binding of the chaperone protein to the coumarin or the coumarin derivative.

13. The method of claim 12, wherein the client protein or the client polypeptide is degraded.